Environmental Health Perspectives
Vol. 99, pp. 269–271, 1993

A Highly Sensitive and Specific Method for Quantitation of *O*-Alkylated DNA Adducts and Its Application to the Analysis of Human Tissue DNA

by Ho-il Kang,[1] Chieko Konishi,[1] Toshio Kuroki,[1] and Nam-ho Huh[1]

Formation and accumulation of O^6-alkylguanine and O^4-alkylthymine in human tissues is possibly the most relevant marker for cancer risk. Because humans are chronically exposed to diverse kinds of chemicals and eventual DNA structural modifications are supposed to be a complex mixture of adducts at very low levels, it is essential to use an assay with extremely high sensitivity and specificity. We have established a quantitation method, called PREPI, for O^6-methylguanine, O^4-methylthymine, and O^4-ethylthymine by the combination of *pre*fractionation by HPLC, ^{32}P-postlabeling, and *im*munoprecipitation. The detection limit was about 1 fmole for all three adducts, enabling us to anlyze about 1×10^{-8} levels as a molar ratio to normal counterpart using 100 μg of DNA. In a pilot experiment, we analyzed 11 peripheral blood samples from healthy volunteers. O^6-Methylguanine was detected in all the cases with a mean value of $2.0 \pm 1.3 \times 10^{-8}$ (range, $0.78–4.6 \times 10^{-8}$). Neither O^4-methylthymine nor O^4-ethylthymine was above the detection limit of 0.8×10^{-8} as a ratio to thymine.

Introduction

O^6-Alkylguanine and O^4-alkylthymine have been demonstrated to be the most relevant lesions among DNA modifications for induction of cancer by *N*-nitroso compounds in diverse experimental systems (*1–3*). Detection of such DNA lesions in human tissues, therefore, is one of the best markers for the biologically relevant exposure to alkylating agents and may eventuallly lead to a better risk assessment (*4–6*). Because DNA modifications in human tissues have been previously demonstrated to be at extremely low levels, the use of an assay with high sensitivity as well as specificity is needed for molecular epidemiological studies on cancer in man. To address this problem, we have established a quantitation method for *O*-alkyl DNA adducts and examined its validity for application to human samples in a pilot experiment.

Materials and Methods

DNA was enzymatically hydrolyzed to nucleoside-3′-monophosphate (3′-MP) using microccocal nuclease and spleen phosphodiesterase (*7*). Each alkylated 3′-MP was fractionated by a reverse-phase HPLC system under essentially the same conditions as previously described (*5*). The alkylated 3′-MP was then phosphorylated by polynucleotide kinase and [^{32}P]ATP according to Reddy et al. (*7*), followed by 3′-dephosphorylation by decreasing the pH to 6.0 (*8*) for better recognition by antibodies. After purification by HPLC, alkylated 5′-[^{32}-P]MP was immunoprecipitated with corresponding monoclonal antibodies [ER-6 for O^6-methylguanine, EM-3-33 for O^4-methylthymine, and ER-01 for O^4-ethylthymine (*9,10*)], and the radioactivity was determined by liquid scintillation counting. Human DNA samples were isolated from peripheral leukocytes of 11 healthy volunteers who had no apparent history of exposure to alkylating agents.

Results and Discussion

Procedure of PREPI

Conditions of each reaction step were optimized using standard DNA treated with *N*-methyl-*N*-nitrosourea or *N*-ethyl-*N*-nitrosourea. The content of O^6-methylguanine (O^6-MetG), O^4-methylthymine (O^4-MetT), and O^4-ethylthymine (O^4-EtT) in the standard DNA was determined by competitive radioimmunoassay using tritiated tracers and antibodies as described (*5*). Completion of DNA hydrolysis was monitored by injecting aliquots onto HPLC. O^6-Methylguanosine-3′-monophosphate, O^4-methylthymidine-3′-monophosphate, O^6-ethylguanosine-3′-monophosphate, and O^4-ethylthymidine-3′-monophosphate

[1]Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Toyko 108, Japan.

Address reprint requests to N-h. Huh, Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan.

could be separated from each other and also from normal 3′-MP under the present conditions, which facilitated independent analysis of each adduct from a given amount of DNA and reduced the amount of [^{32}P]ATP necessary in comparison with the original postlabeling method. After postlabeling, 3′-phosphate was removed to enhance the recognition by antibodies because the monoclonal antibodies used were originally raised against alkylated nucleosides (*9,10*). The second HPLC fractionated was used to remove [^{32}P]ATP and nonspecific radioactive byproducts before final immunoprecipitation. The precise amount of alkylated DNA adducts was determined by comparing a curve obtained by serial dilution of samples with a standard curve.

Sensitivity of the Method

A representative standard curve for O^4-EtT is shown in Figure 1. Standard curves for O^6-MetG and O^4-MetT were essentially similar to that for O^4-EtT (data not shown). One femtomole of each alkylation product could be precisely determined. Therefore, the relative sensitivity (*11*), i.e., the lowest relative modification level to be determined is about 1×10^{-8} as a molar ratio to normal counterpart when 100 μg of DNA is available for analysis (Table 1).

Specificity

Combining several analytical methods with different physicochemical bases is preferable for attaining the high specificity needed, particularly in the case of human samples. The present method is highly specific because each step, i.e., reverse-phase HPLC fractionation, enzymatic reactions including postlabeling, and immunoprecipitation, contribute to increasing the specificity through different physicochemical or molecular mechanisms.

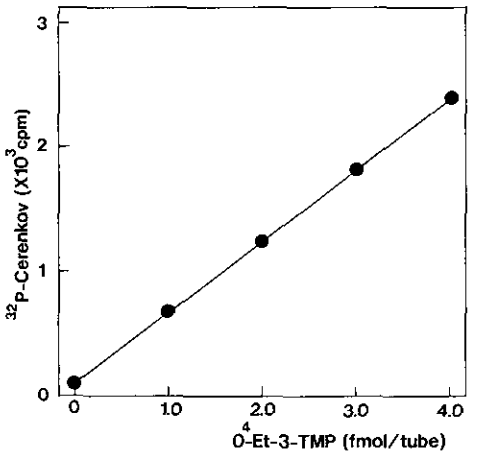

FIGURE 1. A representative standard curve for O^4-ethylthymine. Abscissa shows the amount of O^4-ethylthymine determined by radioimmunoassay. Ordinate is radioactivity of the immunoprecipitates at the final step of PREPI.

Table 1. Comparison of detection methods for O^4-ethylthymine.

Method	Absolute sensitivity, fmole	DNA μg	Relative sensitivity	Specificity
ISB	2	25	1.5×10^{-7}	Moderate
HPLC + RIA	300	20	1.5×10^{-8}	High
PREPI	1	1	1×10^{-9}	Very high
		100	1×10^{-8}	

Abbreviations: ISB, immuno-slot-blot; HPLC, high-pressure liquid chromatography; RIA, radioimmunoassay; PREPI, prefractionation by HPLC, ^{32}P-postlabeling, and immumoprecipitation.

Relevance versus Feasibility

The validity of any analytical methods for use in molecular epidemiological studies with respect to cancer may be assessed by two independent parameters: relevance to cancer development and feasibility or ease of use for mass screening. Quantitation of *O*-alkylated DNA adducts is considered to have higher relevance to carcinogenesis than hemoglobin adducts or N7-alkylguanine, for example. In our previous studies, to detect O^4-EtT in human liver DNA by radioimmunoassay using a tritiated tracer, we had to use more than 20 mg of DNA (corresponding to 20–30 g liver tissue) because of the limited sensitivity. We attempted to improve the feasibility by increasing the sensitivity of the quantitation method without sacrifying the relevance. In the present assay, we need only 100 μg DNA (corresponding to 10 mL of peripheral blood or several hundred milligrams of biopsy tissue specimens) for the analysis of similar relative modification levels.

Analysis of Human Leukocyte DNA

To examine the validity of the present method, we quantitated O^6-MetG, O^4-MetT, and O^4-EtT in peripheral leukocyte DNA obtained from 11 healthy volunteers. O^6-MetG was detected in all 11 cases, with a mean value of $2.0 \pm 1.3 \times 10^{-8}$ (range $0.78–4.6 \times 10^{-8}$) as a molar ratio to guanine. O^4-MetT and O^4-EtT were below the detection limit of 0.8×10^{-8} as a ratio to thymine. The result indicates that humans are exogenously and/or endogenously exposed to methylating agents, and the present method can be practically applied to the analysis of human samples. We are now undertaking studies on a possible correlation between levels of alkylated DNA adducts and the incidence of cancer using the method described above.

This manuscript was presented as a poster at the Conference on Biomonitoring and Susceptibility Markers in Human Cancer: Applications in Molecular Epidemiology and Risk Assessment that was held in Kailua-Kona, Hawaii, 26 October–1 November 1991.

This work is supported in part by a Grant-in-Aid for Special Project Research, Cancer-Bioscience, from the Ministry of Education, Science and Culture, Japan. We thank Manfred F. Rajewsky, Essen University, Germany, for collaboration and for providing the antibodies and tracers.

REFERENCES

1. Goth, R., and Rajewsky, M. F. Persistence of O^6-ethylguanine in rat-brain DNA: correlation with nervous system-specific carcinogenesis by ethylnitrosourea. Proc. Natl. Acad. Sci. U.S.A. 71: 639–643 (1974).
2. Swenberg, J. A., Cyroff, M. C., Bedell, M. A., Popp, J. A., Huh, N., Kirstein, U., and Rajewsky, M. F. O^4-Ethyldeoxythymidine, but not O^6-ethyldeoxyguanosine, accumulates in hepatocyte DNA of rats exposed continuously to diethylnitrosamine. Proc. Natl. Acad. Sci. U.S.A. 81: 1692–1695 (1984).
3. Thomale, J., Huh, N., Nehls, P., Eberle, G., and Rajewsky, M. F. Repair of O^6-ethylguanine in DNA protects rat 208F cells from tumorigenic con-

version by *N*-ethyl-*N*-nitrosourea. Proc. Natl. Acad. Sci. U.S.A. 87: 9883–9887 (1990).
4. Umbenhauer, D., Wild, C. P., Montesano, R., Saffhill, R., Boyle, J. M., Huh, N., Kirstein, U., Thomale, J., Rajewsky, M. F., and Lu, S. H. O^6-Methyldeoxyguanosine in oesophageal DNA among individuals at high risk of oesophageal cancer. Intl. J. Cancer 36: 661–665 (1985).
5. Huh, N., Satoh, M. S., Shiga, J., Rajewsky, M. F., and Kuroki, T. Immunoanalytical detection of O^4-ethylthymine in liver DNA of individuals with or without malignant tumors. Cancer Res. 49: 93–97 (1989).
6. Hall, C. N., Badawi, A. F., O'Connor, P. J., and Saffhill, R. The detection of alkylation damage in the DNA of human gastrointestinal tissues. Br. J. Cancer 64: 59–63 (1991).
7. Reddy, M. V., Gupta, R. C., and Randerath, K. ^{32}P-Base analysis of DNA. Anal. Biochem. 117: 271–279 (1981).
8. Cameron, V., and Uhlenbeck, O. C. 3′-Phosphatase activity in T4 polynucleotide kinase. Biochemistry 16: 5120–5126 (1977).
9. Adamkiewicz, J., Eberle, G., Huh, N., Nehls, P., and Rajewsky, M. F. Quantitation and visualization of alkyl-deoxynucleosides in the DNA of mammalian cells by monoclonal antibodies. Environ. Health Perspect. 62: 49–50 (1985).
10. Eberle, G. M. Monoclonal antibodies against carcinogen-DNA adducts. Doctoral thesis, University of Essen, Essen, Germany, 1988.
11. Huh, N., Moriyama, C., Satoh, M. S., Shiga, J., and Kuroki, T. Detection of O^4-alkylthymine in human liver DNA. In: Immunoassays for Trace Chemical Analysis. Monitoring Toxic Chemicals in Humans, Food and the Environment, ACS Symposium Series 451 (M. Vandelaan, L. H. Stanker, B. E. Watkins, and D. W. Roberts, Eds.), American Chemical Society, Washington, DC, 1990.